

Risk assessment of a genetically modified *Escherichia coli* bacteria to a biosensor for the detection of copper in water for educational and research purposes

Risk assessment dossier of the iGEM Zamorano Project

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Introduction

This document details a risk assessment done to the project "*Know your enemy: Construction of bioscience knowledge*". It consists in developing a biosensor of copper for its use in natural waters contaminated by chemicals such as pesticides. It was developed by genetically modifying the DH5ΔZ1 strain (non-pathogenic) from a *Escherichia coli* bacteria with three different fluorescence color genes (green, pink and orange), using specific promoters for the expression of such genes; which give it a resistance to antibiotics (ampicillin and tetracycline).

To perform the risk analysis of the genetically modified organism previously mentioned, two different approaches were compared: The Cartagena Protocol, proposed by the secretariat itself, and the Guide for the environmental risk assessment of genetically modified organisms (ERA due to its Spanish acronym), developed by fourteen experts in several branches of biotechnology (mostly Latin-Americans). After an in-depth analysis of both methods of assessment, it was decided that the ERA guide was going to be used due to its simplistic application, besides being more didactic and easier to follow than that one of the Protocol. All the studies were performed by the students of the iGEM Zamorano Latin American Team, advised by molecular biology and biosafety specialists, inside Zamorano university's installations located in Honduras. For this reason, biosafety studies are subject to the biosafety regulatory framework of Honduras.

In the educational ambit, knowledge is sought to be imparted to all Zamorano's students, so that they are aware of the benefits that modern biotechnology and all its applications has to offer to the fields of biosensing, bioremediation, bioprevention, among others; all of this, following recombinant DNA protocols that meet expectations of biosafety. Another objective is to raise interest of the Latin American youth towards scientific research through modern biotechnology; so that they can help find effective solutions to various medical, agricultural, industrial and environmental problems. The last objective is to prove that inexperienced youngsters may also make consistent regulations; one that do not hinder the expansion of biotechnology, but that also take the adequate risks into account.

Methodology

The project was developed following the guidelines established by the “Guide for the environmental risk assessment of genetically modified organisms” (Kido et al., 2012). This guide was developed by 14 experts from Latin America in order to outline and facilitate the work of biotechnology regulators. The explanations of the importance of each step are detailed in Chapter I of the same guide, which is available online through the following link:

<http://www.conacyt.mx/cibiogem/images/cibiogem/comunicacion/publicaciones/Guia-evaluacion-riesgo-OGMs.pdf>

Environmental risk assessment (ERA)

Problem formulation of Environmental Risk Assessment in a modified *E. coli*

The main elements that form the context of the DH5ΔZ1 strain of the bacterium *E. coli* are: the legal framework, biology of the bacteria, its genetic construction (as well as other designated changes added to the organism, especially those regarding the possible production of pathogenicity) and leakage of the bacterium in the laboratory. The conditions in which this project is developed (that is, the receiving medium) and the affected organisms are as important as well. There are more than 30 years of scientific reports in favor of using this strain in genetic engineering.

First stage: Definition of context

Legal framework

Honduras signed and ratified the Biosafety Protocol of Cartagena within the Convention of Biological Diversity. As such, it must operate under the articles of the Protocol requiring to develop a risk assessment, among others studies (articles 15, 16, 23 and annex III), for a "Genetically Modified Living Organism" (as it is called in the Protocol).

In Honduras, synthetic biology products, being genetically modified (denominated genetically modified organisms or GMOs), are in the biosafety regulations. Nowadays (July 2014), it is found within the phyto-zoo sanitary law of Honduras, established by Decree No. 157-94, year 1995 (2014 SENASA). However, there is no law or regulation in Honduras that clearly establishes differences between "modern biotechnology" products from synthetic biology products.

In Honduras, GMOs regulation does not require the developer of a genetically modified product, which is confined in a laboratory for educational or research purposes, to conduct a comprehensive risk assessment. However, for this project, a risk assessment was performed following the guidelines of Guide for the environmental risk assessment of genetically modified organisms (Kido et al., 2012). The guide was designed for developers and policy regulators of Latin America as an alternative evaluation method to the one suggested by the Cartagena Protocol in the section "Guidance for risk assessment of Living Modified Organisms, annex III", p. 28, 29, 30 "(Secretariat of the Convention on biological diversity, 2000).

The biosensor of copper risk assessment, carried out by students of the iGEM Zamorano Latin America team, was subjected to the National Committee for Biotechnology and Biosafety of Honduras to obtain authorization to make confine experiments in the laboratory. This was essential to carry out the desired experiments with the organism. The committee granted access for such experiments. This project does not aspire to the commercial release of the same.

Protection goals

- a. To protect the health of those who work in the laboratory; the biosensor based on a genetically modified *E. coli* is used in a laboratory module to teach different techniques and concepts to students. The presentation of students and instructors to this genetically modified organism should not cause damage to their health.
- b. To protect the health of people outside the laboratory practice; avoid at all costs the leakage of the bacterium from the laboratory, and the proliferation of the enterobacteria in humans. The laboratory needs to implement biosafety methods to prevent its escape.
- c. To protect the health of top vertebrates mammals; those who are carriers of the *E. coli* in their digestive tract and which are proven that are susceptible to certain strains of this bacterium. The bacterium should not be able to proliferate in environments other than those required for the practice.

Project Objectives

- a. **Education:** to create a laboratory seminar for students of the University of Zamorano within the program of Learning by Doing. Learning objectives include building capacities at the student in aseptic techniques, basic bacteriology, general principles of biosafety in the laboratory, general principles of genetics and molecular biology, and introduce students to genetic engineering.

- b. **Research:** to develop a biosensor of heavy metal pollution in water, and use it as a biological technique that detects the presence of residues of copper in water. Probably causes could be the improper handling of agrochemicals or residues of mining activities nearby the region. A pollute detection mechanism is the first step to control pollution, to promote a responsible use of these compounds and to preservation of water resources in the area.

Biology of Bacterium

The *E. coli* is the most studied prokaryote organism throughout the history of microbiological science. It was first described in 1885 by Theodor Escherich (Ledermann, 2007). Its family is Enterobacteriaceae, it is anaerobic facultative and negative gram. It possesses a flagellum and it does not create spores. It is widely used in the bio-industry for experiments in molecular biology and genetic replication (ArgenBio, 2010).

Its natural habitat is the intestine of most mammals, and therefore, does not represent a danger to the ecosystem. It takes part in the decomposition of food to facilitate the absorption of nutrients to the host. Its metabolic reactions are known almost completely; it has been determined that the bacterium synthesizes approximately 4400 different proteins, 994 enzymes and 794 metabolites (Martinez & Gosset, 2007).

While it is true that hundreds of strains of *E. coli* are known, only 6 have been identified as pathogenic for humans and superior mammals such as cattle, equines and pig (their outbreaks being well known in the previous years, such as Spain, USA and Germany) (Galli, 2012). These pathogenic *E. coli* contaminate food through contact with fecal coliforms of guests (Moredo, 2012). The main foods that these microorganisms can be found include fruits and vegetables freshly harvested and poorly cooked or improperly refrigerated meats. Pathogenic strains produce a powerful toxin called "Shiga", that stops the protein synthesis of certain cells in the digestive tract and cause diarrheal pictures, urinary tract infection and hemolytic uremic syndrome (WHO, 2005).

Because of its safe historic use in molecular biology, the DH5ΔZ1 strain has been chosen for this experiment. Due to it been non-pathogenic, its use greatly reduces the possible dangers. Likewise, non-pathogenic *E. coli* strains, like K-12 and its derivatives, have been widely used for years by genetic engineering to replicate DNA. The process of replication is made possible by plasmids, which act as vectors of cloning, or expression, to manufacture recombinant proteins on a large scale (like insulin); production of lactate and ethanol from the digestion of sugars

from marine algae; and even production of plastics and enzymes that can be isolated and used in a variety of medical, industrial, and biotechnological processes (Lara, 2011).

In this particular case study, the *E. coli* was modified to activate expression genes of fluorescent colors in the presence of copper. It behaves exactly like any other non-pathogenic *E. coli*, with the exception of a promoter gene that activates the production of specific proteins which enables it to change color. This is what inspired us to re-baptize it as "*E. zamofordl*", referring to the cooperation between Zamorano University and Stanford University, which made the creation of this organism. The rest of the bacterium's metabolism works just exactly as any other not genetically modified, non-pathogenic *E. coli*.

Receiving medium

The bacterium operated completely isolated with in the laboratory. It was cultivated in solid mediums (like petri dishes with agar nutriment gel) and in liquid mediums by controlled growth in broths and solutions. Several practices were executed using these two mediums, obtaining positive results in both.

While it was in storage, it was kept in special refrigerators firmly closed at temperatures between 5-8 ° C and humidity of 45-50%, so that it can enter a controlled state of dormancy. At the time of its use, they were removed and exposed to the laboratory environment (25° C, 50-75% humidity) for a few moments; long enough to take a sample. Then it was returned to the refrigerator on the same physical medium.

The lab is innocuous; presence of animals or insects that could be sources of contamination and spread were not detected. For handling, the investigator or student was required to wear gloves and tweezers to avoid direct contact with the bacterium. No other contact with the bacterium is made by the investigator or student.

Track record of safe use

The *E. coli* is overwhelmingly used by genetic engineering and its recombinant DNA techniques. The techniques emerged in the 1970s as a result of the theoretical knowledge gained in the field of molecular biology. Some even categorized it as a "star body" of the discipline of recombinant DNA (ArgenBio, 2010). The complete genome of the *Escherichia coli* was completely sequenced in February, 1997 (Blattner et al., 1997). The next thing they did was to compare the genome of the K-12, a non-pathogenic strain of *E. coli*, with O157: H7, the most dangerous strain known. It was found that they both share about 3,500 genes, but the pathogenic one possesses 1,300 genes not found in the non-pathogenic bacteria (Blattner et al., 1997).

The DH5ΔZ1 strain is commonly used in many laboratories around the world and can be purchased commercially due to its GRAS classification (Generally Regarded As Safe). DH5ΔZ1 and other strains commonly used today are all derived from the K-12 strain, which is the only one that can be used for molecular biology experiments under NIH guidelines without the need of resorting to strict measures of containment (Jaschke, 2013).

Historically, there have been no reports of negative impacts on human and animal health upon utilization of this strain in biosensing experiments, or other types of experiments, in which the bacteria DH5ΔZ1 have been transformed for the purpose. This background is more than enough proof of the safety of the bacterium. There is no reason to believe that under controlled environments that is going to change.

Second stage: Formulation of the problem and hazards list

The "construction of bioscience knowledge" project has both educational and research purposes. It seeks to detect pesticide residues based on copper in the waters of the coffee zone of Yojoa Lake o nearby mining zones in Honduras. It is possible that because of climate change, the region could have experienced an epidemic of "coffee rust" caused by the phytopathogenic fungus *Hemilea vastatrix*. This could have led the farmers to use more copper based pesticides. Residues left by mines could have leached to nearby water sources.

As part of the detailed study of the *E. zamofordi*, a list of hazards based on all the parameters described in protection goals can be determined. The hazards are as follows:

- a. Horizontal gene transfer to other microbial populations;
- b. Vertical gene transfer to non-genetically modified *E. coli*;
- c. Pathogenicity in humans;
- d. Pathogenicity in other beneficial microorganisms;
- e. Leakage of the bacterium though physical medium (footwear, air, insects);
- f. Leakage of the bacterium through waste disposal areas of the laboratory;
- g. the fixing of the bacterium in the human microbiota;
- h. Invasive competitiveness of the modified *E. coli* upon the *E. coli* naturally found in the environment.

Third stage: Risk characterization

With respect to gene flow (hazards a and b), both are discarded because the bacteria are maintained isolated in their growth media at all times and do not have contact with other strains of *E. coli* or other microorganisms, as they are not released into the environment yet. Furthermore, the DH5ΔZ1 strain does not produce spores, so the anemophilous or hydrophilic drift of genetic material is also discarded.

Regarding the possibility of pathogenicity (hazards c and d), the induced changes in the organism do not involve the use of pathogenicity genes and the promoters that induce color change do not represent toxicity to other life forms as well. Besides, recalling the track record of safe use, no cases in which the DH5ΔZ1 strain has sickened any organism has been reported in the years it has been used in experiments (Jaschke, 2013). Possible mutations that cause these bacteria to produce toxicity, similar to the *E. coli* O157: H7 that produce the "Shiga" toxin is extremely improbable because the pathogenic strain used differs by more than 1300 base pairs in the gene structure, including the genes that target higher vertebrate mammalian (Blattner et al. 1997).

Given the ongoing implementation of the Biosafety Manual in the Laboratory by the WHO, and biocontainment mechanisms, the possibility of leakage of the organism outside the laboratory are reduced to the minimum (hazards e and f). The use of personal protective equipment (PPE), the application of 70% isopropyl alcohol to the medium to kill the bacteria once they have served their purpose, sanitizing the used tools and clothes with bleach solutions at 5% and the subsequent sterilization of them in the autoclave are some of the measures taken to prevent the leakage of bacterium from the laboratory boundaries. The presence of living insects is not allowed under any circumstance. Therefore, the possibility of a leakage because of insects is discarded due to a lack of a logical causal connection. In a given case that the *E. coli* manages to leak out of the laboratory boundaries, maybe because of a student oversight, the modified *E. coli* will not proliferate because the DH5ΔZ1 strain was specially designed to survive under strict controlled medium environment. In other words, the slightest change in temperature, humidity or pH affects the bacterium lethally. That is why the DH5ΔZ1 strain, and other strains derived from the K-12 strain, these are the only ones allowed to be used in molecular biology experiments (Jaschke, 2013).

The possibility that the modified *E. coli* will fix itself within the human microbiota (hazard g) is also discarded because the conditions of the human intestinal tract are very different from the

ones in the culture medium. The medium environment is necessarily required for the development of these. Nor can there be invasive competitiveness (hazard h) as it has been already specified that the bacterium is not able to leave the laboratory. The environment in which an unmodified *E. coli* lives is not favorable for them to develop.

The next graph, entitled "Path to harm", is one of the tools provided by the Guide for the environmental risk assessment of genetically modified organisms. It was used for the development of this Risk Assessment. It aims to show the sequence of steps and conditions that should be met for actual by the modified *E. coli* to occur. All studies ruled out the hazards due to lack of logical causal connection or scientific basis. The risks that were listed as possible were clearly mitigated through biocontainment and biosafety measures.

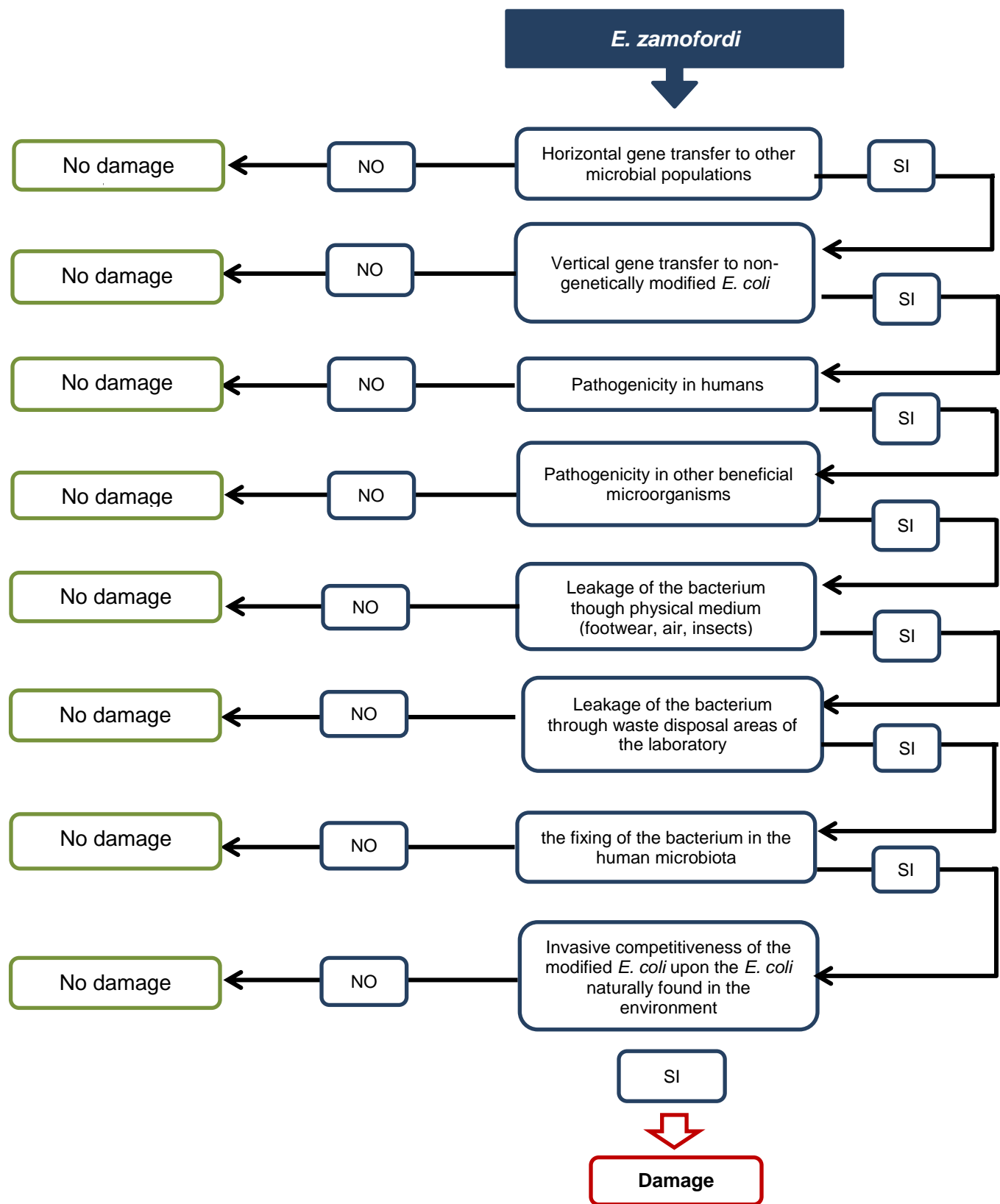


Figure 1. Path to damage analyzing all hypothetical dangers cited. It is concluded that the changes made to the bacterium does not represent any danger to either human health or the environment.

Fourth stage: Risk estimation

Based on the list of hazards created in the last step, we can proceed to the study of the binominal probability/ effect of the exposition for the risk estimation in the context previously established (Figure 2). The considerations below are part of the Path to harm.

Biologically, based on the track record of safe use, the modified bacterium does not represent any danger based in its security record. The genetic transfer flow, pathogenicity and mutations are therefore categorized as very unlikely. This means that the estimated risk is insignificant.

Regarding the handling to avoid a leak of the bacterium out of the laboratory boundaries, there are a series of barriers and biocontention mechanisms that reduce significantly this probability. Upon a flaw in the preventive systems, the organism itself counts with a biological barrier: this *E. coli* strain does not proliferate outside its specifically prepared medium. It is also very susceptible to the natural environment. Given the low probability of occurrence and its consequences, its risk is estimated to be insignificant.

The graphic below is another tool provided by the ERA guide. It helps to qualitatively estimate the risk of handling the DH5ΔZ1 strain of the *E. coli*. It is based upon the information located in the phase of risk characterization.

		Risk estimation			
Probability	Very High	Low	Moderate	High	High
	High	Low	Low	Moderate	High
	Low	Insignificant	Low	Moderate	Moderate
	Very Low	Insignificant	Insignificant	Low	Moderate
		Marginal	Minor	Intermedia	Mayor
		Consequence			

Figure 2. A representation of the total risk estimation based of the characterization of the risk in the context explained before. (Adapted by Souza and Andrade, 2012).

Disposal method

Upon completion of the experiments on the bacterium under study, the ideal environment for its development was destroyed. It was destroyed by adding isopropyl alcohol to the test tube and petri dishes that have had any contact with the organism. The isopropyl alcohol killed all microorganisms in the medium by disintegrating the cell membrane and denaturizing the proteins present within the bacterium thereafter.

Then, these wastes are gathered in special containers. The solid wastes were separated from the liquid ones. They were subsequently removed along with the debris from the Medical University Clinic.

Finally, all the instrumentation used was sanitized with chlorinated water and brushed to maintain asepsis in the laboratory. Then it was subjected to high temperatures and pressures within the autoclave.

The proper management of bio-waste through applying bio-containment procedures is one of the steps to ensure compliance with the established biosafety standards and integrity of the participants.

Annexes

Biosecurity aspects

It was proven, through the risk evolution, that the lab practices do not represent a biological hazard to people. Even so, because the project has educational purpose, Biosafety Level 2 was selected; to teach students aseptic technics and biosafety norms in the lab. All of this following the lineaments established by the WHO in its publication *“Biosecurity manual in the lab”* in 2005.

Risk Group	Biosafety level	Laboratoy type	Laboratory Practice	Security Equipment
1	Basic level 1	Basic education, research.	AMT	None, uncovered lab desk
2	Basic, level 2	Primary attention service; diagnostic, research.	AMT protection clothing; biological risk signal.	Uncovered lab work, and BSC for possible volatile.
3	Containment Level 3	Special diagnostics, research.	Level 2 practices and special clothing, controls access and directional air flow.	BSC along with primary containment methods for all activities.
4	Maximun containment, Level 4	Dangerous pathogen units.	Level 3 practices with entrance chamber and hermetic sealing, flushing exit and special residue disposal.	Class 3 BSC o pressurized suits along with class 2 BSC, double layer autoclave and, air filter.

AMT: Appropriate Microbiology Techniques, BSC: Biological Security Chamber

* *Taken from “Manual de Bioseguridad en Laboratorio”, pag. 15 (OMS, 2005).

Good safety practices

Good practices of safety (GPS) were adapted to the lab practice that was going to be done. To guarantee total safety of the people that worked in the modification of the bacterium, the following biosafety norms in the lab need to be followed:

Access

- Only allowed staff member were allowed to enter the working zones of the lab.
- The lab´s door remained close all time.

- The entry to children or animals in the working zones of the lab was not allowed.

Personal Protection

1. Housecoats were used during the laboratory practices at all times.
2. Protective gloves were used. They were appropriate to all procedures that may imply direct contact or accidents with blood, corporal fluids, and other materials potentially infectious. Once used, the gloves were removed in an aseptic way and following with hand washing.
3. Staff members had to wash their hands after manipulating lab materials and before leaving the working zone.
4. Safety goggles and other protective devices were used when it was necessary to protect the eyes or the face from impacts and ultraviolet radiation sources.
5. Housecoats and other protective equipment and instruments were use exclusively in the authorized areas within the laboratory, in order to maintain innocuity during the practice.
6. There were use shoes with metal point.
7. Eating, drinking, smoking, using cosmetics, or the use of contact glasses in the working zones.
8. Storage food or beverages for human consumption within the working zones of the lab was prohibited.
9. Protective clothes from the lab were not stored in the same lockers as the Street clothes.

Procedures

1. Mouth pipetting was strictly forbidden.
2. No material was placed in the mouth or licked.
3. All technical procedures were performed so as to minimize the formation of aerosols and droplets.
4. In the event of a spill, accident, actual or potential exposure to infectious materials, the supervisor should have been notified immediately. During the development of the project none of the above cases occurred.
5. Contaminated liquids were decontaminated (by chemical or physical means) before their disposal by the collector of sanitation. Furthermore, Zamorano University counts with its own effluent treatment mechanisms within its property, which will be detailed in biocontainment standards.
6. Documents to be written out of the laboratory were protected from contamination while they were in it.

Biocontainment

1. The laboratory was kept neat, clean and free from materials not related to the practice.
2. Given any accidental spills of hazardous materials, the following procedure was the decontamination of the area with alcohol and chlorine. Said incidents did not occur during the practices.
3. All instruments, samples and crops with biological material must have been decontaminated before their disposal. In the scenario of them been reused, they were cleaned and sterilized.
4. Windows that could be opened were equipped with racks that prevented the passage of arthropods.

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